

# CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Regulatory T Cells in the Pathophysiology of Immune Thrombocytopenia

Tetsuya Nishimoto and Masataka Kuwana

Regulatory T cells characterized by CD4<sup>+</sup>, CD25<sup>+</sup>, and transcription factor forkhead box P3, called Tregs, are a subpopulation of CD4<sup>+</sup> T cells specialized for immune suppression. Tregs contribute to maintenance of peripheral immune tolerance, and their defects are thought to play a role in the pathogenesis of various autoimmune diseases. Immune thrombocytopenia (ITP) is an autoimmune disease characterized by increased platelet destruction and reduced platelet production, resulting in decreased platelet count. Recently, a series of studies in adults and children with ITP have found that the frequency of Tregs is reduced in circulation, bone marrow, and spleen, and Treg function is impaired. Treg dysregulation is improved after platelet count is recovered by treatment with dexamethasone, rituximab, or thrombopoietin receptor agonists. In addition, a critical role of Tregs in preventing the anti-platelet autoimmune response has been demonstrated in mice deficient in functional Tregs. Thrombocytopenia observed in Treg-deficient mice is mediated through production of IgG anti-platelet autoantibodies, which is analogous to human ITP. Further studies evaluating mechanisms of Treg dysregulation in ITP patients are necessary to elucidate the pathogenesis of ITP and develop novel therapeutic strategies that suppress anti-platelet autoimmune response.

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Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder in association with increased platelet destruction and impaired platelet production, both of which are mediated by IgG anti-platelet autoantibodies.<sup>1</sup> The major autoantibody targets are platelet membrane glycoproteins (GPs), such as GPIIb/IIIa and GPIb/IX.<sup>2</sup> It is widely appreciated that production of IgG autoantibodies requires activation of autoantigen-specific CD4<sup>+</sup> T cells. In fact, in ITP patients, we have identified CD4<sup>+</sup> T cells reactive to GPIIb/IIIa, which are able to stimulate B cells to produce IgG antibodies that bind normal platelet surfaces.<sup>3</sup> Based on detailed analyses of GPIIb/IIIa-reactive CD4<sup>+</sup> T cells and B cells in ITP patients, we have proposed a “pathogenic loop” model for the ongoing IgG anti-platelet autoantibody

response in ITP patients (Figure 1).<sup>4</sup> Specifically, macrophages in the reticuloendothelial system capture opsonized platelets via Fcγ receptors, and present antigenic platelet GP-derived peptides to T cells. Autoreactive CD4<sup>+</sup> T cells are then activated by recognition of the antigenic peptides and exert helper activity to stimulate B cells to produce IgG anti-platelet autoantibodies, which in turn bind to circulating platelets. Theoretically, once this pathogenic loop is established, production of IgG anti-platelet autoantibodies goes on endlessly.

It is likely that the immune system is repeatedly exposed to foreign proteins that cross-react with autoantigens. In this regard, onset of ITP often precedes virus infection or immunization, especially in children. In addition, IgG anti-GPIIb/IIIa antibodies in patients with human immunodeficiency virus (HIV)-related ITP cross-react with HIV-associated gp120.<sup>5</sup> These potentially cross-reactive foreign antigens may trigger the pathogenic loop, resulting in continuous production of IgG anti-platelet autoantibodies. Thus, immune regulatory mechanisms should play a critical role in preventing potential harmful autoimmune response to circulating platelets. One of the immune regulatory mechanisms involved in the pathogenic process of ITP is an inhibitory Fcγ receptor FcγRIIB expressed on reticuloendothelial macrophages. Namely, platelet

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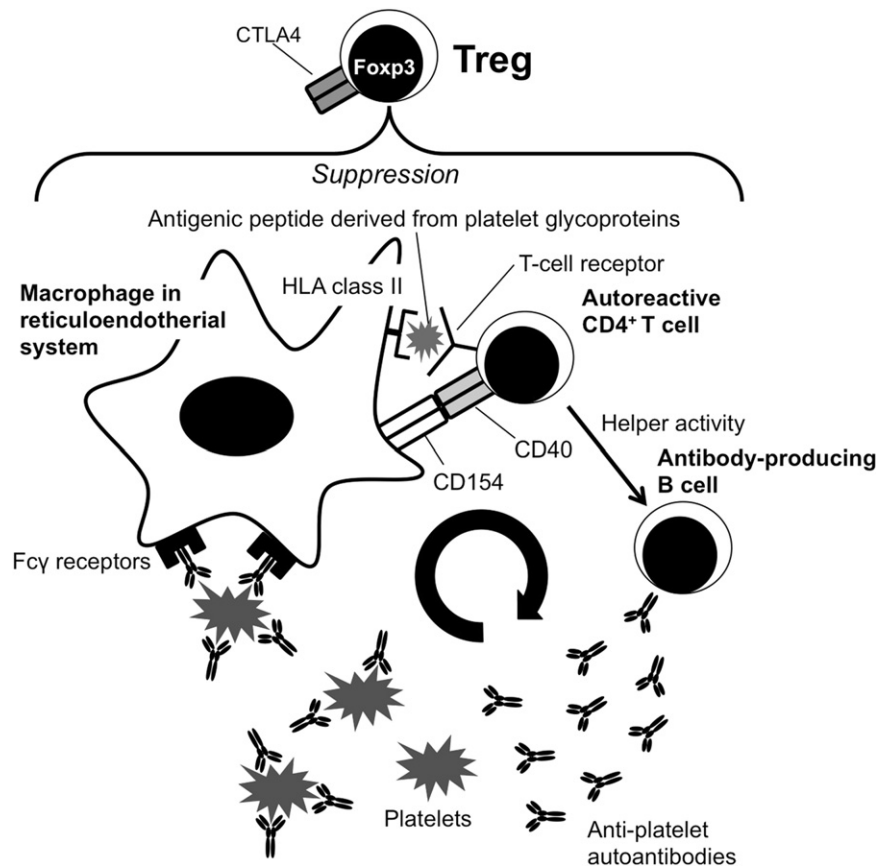
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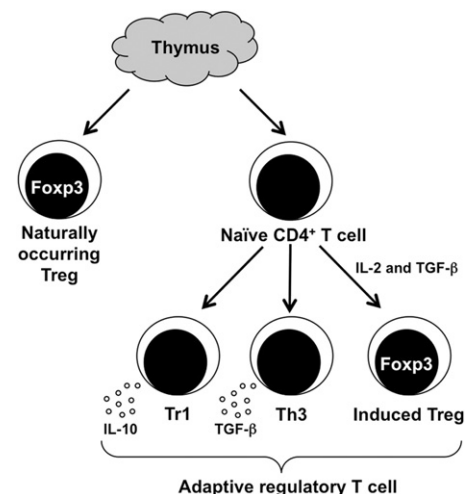
**Figure 1.** Schematic representation of a continuous pathogenic loop carried out by macrophages in the reticuloendothelial system, autoreactive CD4<sup>+</sup> T cells, and antibody-producing B cells that maintains anti-platelet autoantibody production in ITP patients. Tregs suppress an interaction between antigen-captured macrophages and autoreactive CD4<sup>+</sup> T cells, resulting in prevention from a drive of the pathogenic loop.

recovery observed in ITP patients after eradication of *Helicobacter pylori* is mediated through a change in Fcγ receptor balance toward the inhibitory FcγRIIB.<sup>6</sup> Another potential immune regulatory mechanism includes maintenance of immune tolerance and homeostasis in periphery by CD4<sup>+</sup> regulatory T cells.<sup>7</sup> Of various CD4<sup>+</sup> regulatory T cell subsets, CD4<sup>+</sup> T cells with high expression of CD25 and expression of transcription factor forkhead box P3 (Foxp3), called Foxp3 Tregs or just simply called Tregs, have recently been examined for their potential association with pathophysiology of ITP. In this review, we summarize updated knowledge of roles of Tregs in the pathogenic process of ITP.

### CD4<sup>+</sup> REGULATORY T-CELL SUBSETS

CD4<sup>+</sup> Tregs directly suppress acquired immune responses in periphery and are essential regulators of self-tolerance.<sup>7</sup> They are a heterogeneous cell population with distinct surface phenotypes, cytokine production profiles, and mechanisms of immune suppression. Figure 2 summarizes well-characterized subsets of CD4<sup>+</sup> Tregs. Historically,

Sakaguchi and colleagues reported an intriguing finding that neonatal thymectomy 3 days after birth in mice resulted in autoimmune damage of various organs, such as thyroid gland, stomach, ovary, and testis, with appearance of tissue-specific



**Figure 2.** Distinct subsets of CD4<sup>+</sup> regulatory T cells. Tr1, type 1 regulatory T cells; and Th3, T helper 3 cells.

autoantibodies in circulation.<sup>8</sup> This led to discovery of autoimmune suppressive T cells, which were produced in the thymus and delivered to the periphery. This regulatory T-cell subset with expression of CD25 and FoxP3 is currently called naturally occurring Tregs. On the other hand, CD4<sup>+</sup> T cells that acquire regulatory properties under particular conditions in periphery are called adaptive Tregs, which include type 1 Tregs (Tr1) that produce a high level of interleukin (IL)-10,<sup>9</sup> and T helper 3 cells (Th3) that produce transforming growth factor- $\beta$  (TGF- $\beta$ ).<sup>10</sup> Interestingly, CD4<sup>+</sup> Tregs with identical phenotype to naturally occurring Tregs can be generated by antigenic stimulation of naive CD4<sup>+</sup> T cells in the presence of TGF- $\beta$  and IL-2.<sup>11-14</sup> This adaptive regulatory T-cell subset is called induced Tregs. We focus on CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs, which include both naturally occurring and induced Tregs, in this review.

Mechanisms of immune suppression by Tregs are not fully elucidated, but several distinct mechanisms are proposed to date.<sup>15,16</sup> First, Tregs secrete immunosuppressive cytokines, such as TGF- $\beta$ , IL-10, and IL-35. Second, Tregs exert direct cytotoxicity to activated effector T cells through secretion of perforin and granzyme A. Third, Tregs inactivate effector T cells via cell surface immunosuppressive molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA4) and Fas ligand. Moreover, CTLA4 expressed on Tregs modulates functions of specialized antigen-presenting cells, such as dendritic cells, through binding to CD80/CD86, resulting in inhibition of maturation, downregulation of CD80/CD86, and induction of immunoregulatory enzyme indoleamine-2,3-desoxygenase. Finally, Tregs compete with effector CD4<sup>+</sup> T cells for interaction with antigen-captured antigen-presenting cells.

## TREGS AND AUTOIMMUNE DISEASES

The negative selection process in the thymus deletes T cells reactive to autoantigens expressed by thymic epithelial cells, and plays a critical role in maintenance of the central immune tolerance. On the other hand, autoreactive T cells that escape from apoptosis during the negative selection process are delivered to periphery. These potentially harmful autoreactive T cells are suppressed or deleted in periphery through Treg-mediated mechanisms.<sup>15</sup> If this were true, impairment of Treg-mediated immune regulation would lead to development of autoimmune diseases. In fact, mice and humans with genetic mutations in the *Foxp3* gene, which lack functional Tregs, represent severe inflammation and autoimmunity, indicating importance of Tregs in preventing harmful autoimmune response.<sup>17</sup> In addition, reduced number of Tregs and defect in Treg

function have been reported in patients with various autoimmune diseases, including type 1 diabetes, multiple sclerosis, and systemic lupus erythematosus.<sup>18</sup> Interestingly, gene polymorphisms within the *CTLA4* locus have been identified as the disease susceptibility gene for many autoimmune diseases, including type 1 diabetes, myasthenia gravis, systemic lupus erythematosus, and rheumatoid arthritis.<sup>19</sup>

## DYSREGULATED TREGS IN ITP PATIENTS

Given a critical role of Tregs in preventing the autoimmune response, dysregulation of Tregs could also be associated with pathophysiology of ITP. Since 2007, many investigator groups examined frequency and function of Tregs in patients with ITP (Table 1).<sup>20-31</sup> The majority of studies demonstrated a decreased frequency of Tregs in peripheral blood CD4<sup>+</sup> T cells in ITP patients, compared with healthy controls. The lowest Treg frequency was detected in patients with acute phase of the disease and/or with low platelet count, and Tregs were increased in remission. Treg deficiency was reported in both adults and children with ITP. One study in children found that severe reduction of Tregs was associated with prolonged thrombocytopenia, while mild reduction was associated with brief duration, suggesting that Treg proportion may be a prognostic marker.<sup>30</sup> Some studies failed to detect differences in Treg frequencies between ITP patients and healthy controls. These inconsistent results may be explained by use of different phenotypes for identification of Tregs (CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>Foxp3<sup>+</sup>, CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup>, CD4<sup>+</sup>CD25<sup>bright</sup>, and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>). For example, three studies that used CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> cells to define Tregs failed to detect difference in the Treg frequency in peripheral blood from ITP patients and healthy controls.<sup>24,26,29</sup> Interestingly, cells with this phenotype abundantly contain naturally occurring Tregs, suggesting that quantitative alternation occurs primarily within induced Tregs, rather than naturally occurring Tregs. It is imperative to take account of definitions of Tregs upon interpretation of the results in individual studies. On the other hand, Treg's ability to suppress allogeneic T-cell response was assessed by a co-culture assay consisting of sorted Tregs and allogeneic effector CD4<sup>+</sup> T cells. In all studies evaluating Treg function, their immunosuppressive function was inferior in ITP patients than in controls.<sup>21,23,24,26</sup>

Treg frequency was also decreased in bone marrow and spleen from patients with ITP.<sup>25,29</sup> A recent histologic analysis of spleen from ITP patients identified two different splenic structures accommodating proliferating B cells; germinal center and

**Table 1. Frequency and Function of Tregs in Periphery From ITP Patients**

	Subjects of ITP	Method for Identification of Tregs	Treg Frequency*	Treg Function*	Therapeutic Responses of Treg Frequency and Function
Sakakura M et al <sup>20</sup>	Adults	CD4 <sup>+</sup> CD25 <sup>+</sup> (FCM)	Decreased in PB	NT	NT
Liu B et al <sup>21</sup>	Adults	CD4 <sup>+</sup> CD25 <sup>+</sup> (FCM)	Decreased in PB	Defective in PB	NT
Ling Y et al <sup>22</sup>	Adults	CD4 <sup>+</sup> Foxp3 <sup>+</sup> (FCM)	Decreased in PB	NT	Restored frequency with high-dose dexamethasone
Stasi R et al <sup>23</sup>	Adults	CD4 <sup>+</sup> Foxp3 <sup>+</sup> (FCM)	Decreased in PB	Defective in PB	Restored frequency and function with RTX
Yu J et al <sup>24</sup>	Adults	CD4 <sup>+</sup> CD25 <sup>high-</sup> Foxp3 <sup>+</sup> (FCM)	Unchanged in PB	Defective in PB	NT
Olsson B et al <sup>25</sup>	Adults	CD4 <sup>+</sup> CD25 <sup>bright</sup> (FCM)	Unchanged in PB Decreased in BM	NT	NT
Bao W et al <sup>26</sup>	Adults	CD4 <sup>+</sup> CD25 <sup>high-</sup> Foxp3 <sup>+</sup> (FCM)	Unchanged in PB	Defective in PB	Restored function with thrombopoietic agents
Li Z et al <sup>27</sup>	Adults	CD4 <sup>+</sup> CD25 <sup>+</sup> C-D127 <sup>low</sup> (FCM)	Decreased in PB	NT	Restored frequency with RTX plus glucocorticoids
Aboul-Fotoh Lel-M et al <sup>28</sup>	Children	CD4 <sup>+</sup> CD25 <sup>high</sup> (FCM)	Decreased in PB	NT	NT
Audia S et al <sup>29</sup>	Adults	CD4 <sup>+</sup> CD25 <sup>high-</sup> Foxp3 <sup>+</sup> (FCM)	Unchanged in PB Decreased in spleen	NT	Unchanged frequency with RTX
Zahrán AM et al <sup>30</sup>	Children	CD4 <sup>+</sup> CD25 <sup>high-</sup> Foxp3 <sup>+</sup> (FCM)	Decreased in PB	NT	NT
Daridon C et al <sup>31</sup>	Adults	Foxp3 <sup>+</sup> (IHC)	Decreased within GC PLN of spleen	NT	NT

\*Compared with healthy or non-ITP controls.

Abbreviations: ITP, immune thrombocytopenia; FCM, flow cytometry; IHC, immunohistochemistry; PB, peripheral blood; BM, bone marrow; GC, germinal centers; PLN, proliferative lymphoid nodules; NT, not tested; RTX, rituximab.

proliferative lymphoid nodule.<sup>31</sup> Foxp3<sup>+</sup> Tregs were reduced within these two structures, suggesting involvement of Treg deficiency in activation of autoantibody-producing B cells. Taken together, in ITP patients, Treg deficiency is found in circulation as well as in the secondary lymphoid organs.

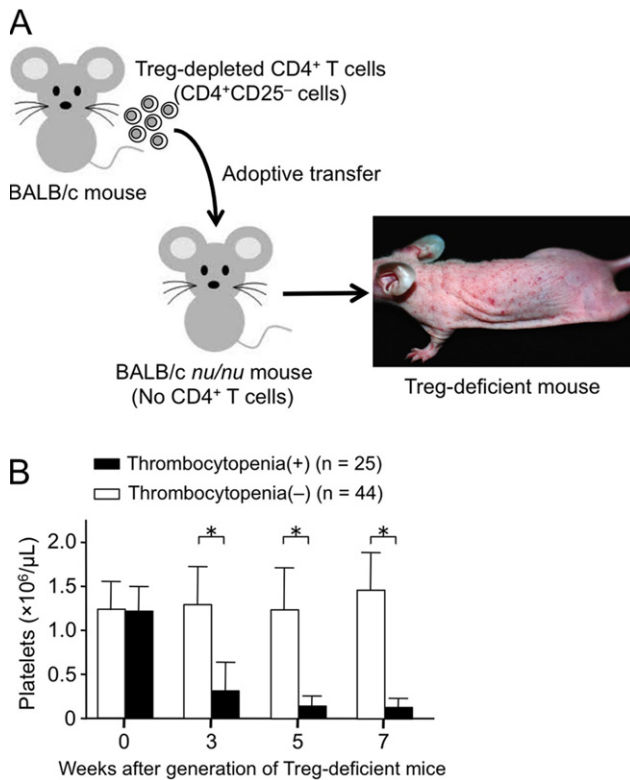
Several studies evaluated serial changes of Treg frequency and function before and after treatment. High-dose dexamethasone and rituximab increased proportion of Tregs in responders,<sup>22,23,27</sup> although one study failed to confirm an increase of Treg frequency after rituximab treatment.<sup>29</sup> On the other hand, thrombopoietin receptor agonists failed to increase Treg frequency, but improved Treg

function.<sup>26</sup> Plasma levels of TGF- $\beta$ , which is required for development of induced Tregs, were increased after treatment with thrombopoietin receptor agonists.

## ROLES OF TREGS IN PATHOPHYSIOLOGY OF ITP

To investigate Tregs' role in preventing ITP, we examined whether Treg deficiency induced ITP in mice. Treg-deficient mice were generated by transferring Treg-depleted CD4<sup>+</sup>CD25<sup>-</sup> T cells isolated from BALB/c splenocytes into syngeneic nude mice (Figure 3).<sup>32</sup> Three weeks after transfer, some mice





**Figure 3.** Autoantibody-mediated immune thrombocytopenia in Treg-deficient mice. (A) Treg-deficient mice were generated by adoptive transfer of Treg-depleted CD4<sup>+</sup> T cells isolated from BALB/c mice into syngeneic nude mice. Some Treg-deficient mice developed bruises in association with a low platelet count (inset). (B) Serial platelet counts in Treg-deficient mice with and without thrombocytopenia. \* $P < .01$ .

spontaneously developed bruises in association with a low platelet count. When the number of Treg-deficient mice was increased, we confirmed that approximately one third of them developed chronic thrombocytopenia, which sustained for at least 12 weeks. IgG antibodies capable of binding to intact platelets were exclusively detected in platelet eluates and in supernatants of unstimulated splenocyte cultures from thrombocytopenic mice. These findings suggest that thrombocytopenia observed in Treg-deficient mice is mediated through production of IgG anti-platelet autoantibodies, which is analogous to human ITP. Furthermore, Treg-deficient mouse is a novel animal model for ITP, especially reflecting induction and propagation of the autoimmune response to platelets.

To confirm the theory that Treg deficiency was responsible for the onset of ITP, CD4<sup>+</sup>CD25<sup>+</sup> Tregs were transferred together with Treg-depleted CD4<sup>+</sup> T cells. As expected, simultaneous transfer of Tregs completely prevented the onset of ITP, while adoptive Treg transfer after onset of ITP had no effect on platelet count, suggesting a primary role of Tregs in induction phase rather than maintenance phase of the autoimmune response. We further examined

mechanisms responsible for Tregs' effects on suppression of the ITP onset, focusing on CTLA4. Treatment with anti-CTLA4 blocking antibody cancelled the Treg's protective effect against ITP, but treatment with control antibodies had no effect. Taken together, results obtained from our Treg-deficient ITP model indicate that Tregs play a critical role in preventing anti-platelet autoimmune response by engaging CTLA4.

Recently, mechanisms for peripheral Treg deficiency were investigated using another mouse model for ITP. In this model, GPIIIa knockout mice were immunized with wild-type platelets, and their splenocytes were transferred into severe combined immunodeficiency mice. This treatment resulted in onset of thrombocytopenia, production of IgG anti-platelet antibodies, and induction of platelet-reactive cytotoxic T cells.<sup>33</sup> Interestingly, in this ITP model, Tregs in the thymus were markedly increased, but Tregs in the spleen were decreased, compared with control mice.<sup>34</sup> Treatment with intravenous immunoglobulin raised the platelet count, and normalized the Treg distribution in the thymus and spleen. These results propose an intriguing theory that peripheral Treg deficiency is caused by thymic retention.

## CONCLUSIONS AND FURTHER PROSPECTS

Recent studies in patients with ITP and in mouse models mimicking ITP have clearly shown that Tregs play an essential role in preventing from a drive of a "pathogenic loop" model for the pathophysiology of ITP (Figure 1). Treg impairment allows autoimmune effector mechanisms to elicit ITP, upon exposure to the trigger known to induce ITP, such as infection of certain microorganisms. Further studies evaluating mechanisms for Treg dysregulation in ITP patients are necessary to elucidate the induction phase of pathogenesis of ITP. On the other hand, two recent studies have shown that low-dose IL-2 therapy leads to recovery and activation of Tregs and subsequent clinical improvement in patients with chronic graft-versus-host disease or in those with vasculitis related to hepatitis C virus infection.<sup>35,36</sup> Although adoptive transfer of Tregs after onset of the disease is shown to be inefficient in various autoimmune mouse models, low-dose IL-2 therapy is a promising approach to develop a novel therapeutic strategy for autoimmune diseases, including ITP.

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